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Impact of Paraquat on Living Systems: The Magic Bullet that Releases Superoxide Radicals

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ABSTRACT
The impact of toxic Reactive-Oxygen-Species (ROS) generated by the herbicide paraquat in aerobic cells have a profound consequence on its survivability, especially when these cells lack anti-oxygenic enzymes. The present study shows a significant decrease in % survivability even in wild-type-facultative-bacteria such as *Salmonella typhimurium,* to as much as 44.4% when exposed to 500 µM paraquat. At 1000 µM paraquat concentration, none of the 4 strains that were examined survived. These were the wild-type *S. typhimurium* virulent 2000 strain, QC-779 which are *Escherichia coli* cells deficient in both Mn and Fe Superoxide-Dismutase (SOD), pDT-1.22 cells which are QC-779 *E.coli* double-mutant-cells transformed with the cloned Mn SOD gene from a strain of *E.coli* GC-4468, and pSSS5* cells which are QC-779 *E.coli* double-mutant-cells transformed with the cloned Mn SOD gene from the *S. typhimurium*-virulent-2000-strain. Paraquat concentration, as low as 50 µM was sufficient to cause a dramatic fall in % survivability to 4.8 % in QC 779 cells. On doubling the paraquat concentration to 100 µM, % survivability of QC-779 cells remained at 4.8%, but that of pDT 1.22 cells and pSSS5* cells dropped to 6.9% and 13.9% respectively from 83.3% and 75.8% respectively. The 2 fold increase in % survivability in pSSS5* cells compared to the pDT 1.22 cells is suggestive of the differential functionality of the Mn SOD gene from *S. typhimurium* and *E.coli* cells respectively, although their origin is from bacterial cells belonging to the same family *Enterobacteriaceae*. This observation is of significance, as it clearly suggests that the *S. typhimurium-2000*-strain and *E.coli* GC-4468 strain must exhibit differential virulence in their native state. It could be a direct consequence of more free-radicals being generated by the *S. typhimurium* 2000 strain versus *E.coli* -GC-4468 cells. This could possibly be manifested in the form of a better defence mechanism in the former, as elicited by a greater amount of SOD production to detoxify the harmful free-radicals within the cell. On increasing the paraquat concentration to 250 µM, only the wild-type *S. typhimurium*-virulent-2000 -cells survived, although it's % survivability fell to half of what was observed when no paraquat was administrated in the growth-media of the bacteria. The protective role of Mn SOD in the cell is thus suggestive of Primordial Importance. This argument could be substantiated by the classic evidence that ROS originate in the mitochondria, during the process of aerobic-respiration. It holds good, in spite of the recent findings that both *S. typhimurium* virulent *2000* cells and *E.coli*-QC-779 cells which are derivatives originally from the GC 4468 *E.coli* cells clearly exhibit Cu/Zn SOD activity in their periplasm.

Keywords: Oxidative-Stress, Reactive-Oxygen-Species (ROS), Mn Superoxide-Dismutase (Mn SOD), Paraquat, wild-type *S. typhimurium* -virulent-2000, *QC 779- E.coli*-double-mutants, pDT1.22 and pSSS5*

INTRODUCTION

The role of oxygen in living systems has always been intriguing, in spite of its impact on Oxidative-Stress having been established more than three decades ago [1]. Although less than 2% of O_2 consumed by living organisms results in the forming of intermediate states of reduced-oxygen, it has proved to be toxic to the cell by causing lipid-peroxidation and DNA-damage, resulting in various neuro-degenerative diseases such as Parkinson's disease, Alzheimers and Amyotrophic lateral sclerosis (AMS) [2] & [3]. However, the exact mechanism by which oxygen precisely results in cell damage is still an important focus of research.

Recent studies on the role of anaerobic conditions on living cells have clearly shown that there is minimal impact on percentage survivability. In fact, these cells were found to survive even better than those compared to cells grown in aerobic conditions [4]. The goal of the present study is therefore to determine how $O_2^{\bullet-}$ radicals produced by paraquat exclusively have an impact on the % survivability in *in-vitro* conditions. The intermediate states of reduced-oxygen which are predominantly toxic to living systems as a consequence of oxidative-stress are listed below as a result of step-wise transfer of electrons to oxygen [5], [6] & [7].

1. $O2 + e' \rightarrow O_2^{\bullet-}$
2. $O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2$
3. $H_2O_2 + O_2^{\bullet-} \rightarrow OH^{\bullet} + OH^- + O_2$

Experimentally these toxic-free radicals can be generated within living cells by utilizing a potent herbicide called paraquat (also commonly referred as methyl-viologen) that was widely used all over the world for broad-leaf weed control [8]. It was primarily used to improve crop management by raising the quality of the final harvest products and by decreasing the risk of cross-infection from fungi and insects [9]. Paraquat essentially functions as a magic-bullet, acting as a redox-chemical that abstracts electrons from the electron transport chain or serves as the primary electron acceptor of Photosystem I in the presence of light [8]. The reduction of paraquat was found to be coupled with the release of toxic superoxide radicals ($O_2^{\bullet-}$) within the cells [10]. In spite of paraquat's world-wide usage that brought substantial benefits to food production and sustainable agriculture [11], of late this herbicide is branded as one of the world's worst poisons on the earth [12], and therefore actually banned in several countries [13]. This is primarily because of the translocation within the plant [12], and has proved to be highly toxic for humans and animals, leading to acute poisoning and death [14] & [15], even

if present in trace amounts (<0.2 ppm) as reported to be detectable in the foliage of certain crops such as sugar-beet and cereal [16]. Yet, the use of paraquat in experimental studies in miniscule quantitates, by using extreme caution and protection, is over-ruled by the potential hazards this herbicide could impose on man. The primordial reason therefore, for utilizing paraquat in spite of its obvious draw-backs, is because it readily catalyses the formation of Reactive Oxygen Species (ROS) within aerobically living cells [10]. Over-production of ROS however can damage cell-membrane through peroxidation of membrane-polyunsaturated fatty-acids. The activity of these ROS is countered by intra-cellular anti-oxidant defences provided by Superoxide dismutase (SOD), Catalase, Glutathione Peroxidase/Reductase and Ascorbate Reductase [17].

We have also shown that there is up to 7 fold increase in Superoxide dismutase and as much as 40 fold increase in Catalase in *N. crassa* mycelia when exposed to paraquat, as exhibited by native-gels stained specifically for these two anti-oxygenic enzymes [18] & [19]. Furthermore, our recent findings utilizing two-dimensional IEF-SDS/PAGE as a tool clearly exhibited induced responses to oxidative-stress caused by paraquat-exposure to *Neurospora crassa*. These 2D IEF-SDS/PAGE gels of *N. crassa* total proteins induced by paraquat that were silver-stained, clearly exhibited 3 important features with specific reference to the anti-oxygenic enzymes, Catalase and SOD. Firstly, the subunits of both these enzymes clearly showed heterogeneity, exhibiting a range of pIs. Secondly, these subunits were clearly induced in paraquat-treated *N. crassa* cells, as shown by their increased intensity [19]. Thirdly, several gene-products in the mycelia of *N. crassa* have shown to be up-expressed as elicited by oxidative-stress caused by treatment with superoxide-generating chemicals such as H_2O_2, menadione, and plumbagin, besides paraquat. Clearly, these up-induced proteins were not found to overlap with each other (Chary and Natvig unpublished data).

In addition to the in-built defence mechanisms to protect living cells under oxidative-stress, there are several non-enzymatic active-oxygen scavengers that can play a major-role in controlling these ROS to be kept under bay, when the cells are treated from external sources. These include, Glutathione, α -tocopherol, Lycopene and Ascorbate as scavengers for detoxifying $O_2^{\bullet -}$ within the cell and Mannitol as a scavenger for $OH^{\bullet}$ [20]. In micro-organisms such as *Salmonella typhimurium* and *Escherichia coli* (Family: *Enterobacteriaceae*) which are small gram negative rods; these are facultative anaerobes that function aerobically in the presence of oxygen, but also live by fermentation in anaerobic conditions. With this knowledge in view, firstly it is important to study the % survivability that serves as a direct indicator for aging and subsequent death. Secondly, the induction of anti-oxygenic enzymes in response to various forms of oxidative-stress on most organisms can be addressed by examining these two microbes as model-systems in the laboratory, in the presence and in the absence of oxygen.

MATERIALS AND METHODS

Bacterial Strains - Their Growth and Preservation

One wild-type strain of gram negative rod was chosen for the current study, namely; a virulent wild-type strain of *S. typhimurium* virulent 2000. A double mutant strain of *E. coli* referred to as QC779 that lacked the Mn and Fe Superoxide-dismutase genes, was obtained from Carlioz and Touati [21]. Finally, the QC779 strain were transformed with pDT1.22 and pSSS5* individually. pDT1.22 was the cloned MnSOD gene from *E.coli* GC4468 and pSSS5* was the cloned MnSOD gene from *S. typhimurium*, that was obtained from our laboratory (Chary *et al.*, manuscript in preparation). All these strains were grown on LB medium and stored at -70° C as glycerol stocks. For the growth of these cells in media containing various concentrations of paraquat (50, 100, 250, 500 and 1000 μM) they were plated on LB medium with this herbicide, and the inverted petri-dishes were placed in an incubator at 37°C overnight.

Colony counting, Plotting of Graph and analysis of Data

Colonies were counted manually. In all the cases the number of colonies present in the petri-plates aerobically was considered as 100% survivability. Utilizing these values as the bench-mark, the percentage survivability under paraquat-treated conditions were determined individually, for each of the strains using the formula: (No. of cells grown in media containing different paraquat concentrations / No. of cells grown in media without paraquat) x 100 = % survivability of cells grown in the respective paraquat concentrations.) The results were tabulated in Table 1 and these values were utilized to plot both line-graphs (Fig.1A) and histograms (Fig.1B) by using Microsoft Excel. Subsequently the % survivability-range in the wild-type strain of *S. typhimurium,* QC779 double-mutant strain, pDT1.22 and pSSS5* cells were compared. This data was utilized for analyses and eventually to throw-light on the role of ROS in aerobic cells, generated specifically by paraquat during respiration.

RESULTS AND DISCUSSION

The present study is to identify the role of $O_2^{\bullet -}$ radicals, specifically generated by paraquat, *in-vivo*, on bacterial cells such as *S. typhimurium* virulent 2000 strain, QC779 a double-mutant of Fe and Mn SOD,

transformed QC779 cells with MnSOD cloned from the virulent strain of *E. coli* GC4468, referred to here as pDT1.22 and the transformed QC779 cells with MnSOD cloned from the S. *typhimurium* virulent 2000 strain, referred to here as pSSS5*. Our results showed that at least four crucial factors are inter-related when cells are exposed to oxidative-stress, specifically induced by paraquat. These include: 1) the micro-environment in which the microbes grow; namely the LB medium supplemented with various concentrations of paraquat. 2) The capacity of virulence exhibited in the above media, individually by each the 4 different strains of micro-organisms utilised in this study. 3) The final outcome as manifested in sustainability as % survivability; a visible yard-stick to 4) The eventual protection offered by defence-mechanisms to the cell; predominantly as anti-oxygenic enzymes.

Elaborating on the above issues; Firstly, the micro-environment in the present study is heavily influenced by various concentrations of paraquat that were added to the growth media. It is particularly important to emphasize in this juncture that other toxic chemicals such as plumbagin, menadione and H_2O_2, also release intermediate-states-of-Reduced-Oxygen within the cell, similar to those released on exposure to paraquat. As a consequence the cell undergoes severe oxidative-stress. However, the cell elicits varied responses in the number and amount of the proteins induced to each of these toxic-chemicals individually. Thus, the proteins that are induced as a manifestation for protection of the cell, do not necessarily overlap as an eventuality of the cell being exposed to these varied chemicals (Chary and Natvig unpublished data). In contrast, this up-expression of several proteins that are clearly unique in response to each of the specific toxic-chemicals such as paraquat, menadione, plumbagin and H_2O_2 in *Neurospora crassa* was evident. A myriad of induced proteins were visualized after separation by the utilization of 2- Dimensional IEF/SDS PAGE and subsequently silver-staining the gels [18] & [19]. Similar findings were also observed even in some members of *Enterobacteriacea* (Chary *et al*., manuscript in preparation).

Secondly, based on the oxidative-stress levied by each of the above toxic-chemicals individually, on 2 or more varied micro-organisms of *Enterobacteriaceae*, we have clearly exhibited a differential response in terms of their virulence, given that the yard-stick for this inference is manifested in the % survivability of these individual species (Chary *et al*., manuscript in preparation). This could eventually be attributed to the differential amounts of protective anti-oxygenic enzymes produced by the cell, especially *Superoxide dismutase*, which is the first line of defence. A corollary to this point was clearly shown by Battistoni *et al*.,[22] wherein increased periplasmic Cu/Zn SOD in *E. coli* cells exhibited enhanced survival. Furthermore, there is evidence to show that with regulatory and structural differences in the Cu/Zn SODs of *Salmonella enterica*, it could have an impact on the virulence of the organism ([23]. Our present study also offers support to these findings. As shown in Table 1 and Figs.1A and 1B; at 100µM paraquat concentrations, first, although the same *E.coli* SOD double-mutant cells were transformed by a cloned Mn SOD gene, the fact that pDT1.22 having originated from the *E.coli* virulent GC4468 strain and pSSS5* being from a *Salmonella typhimurium* 2000 virulent strain clearly shows a difference in the % survivability of these two transformed cell-types. Furthermore, the pSSS5* cells exhibited a two-fold increase in survivability on comparison to the pDT1.22 cells. This two-fold increase in survivability suggests a corresponding two-fold increase in the amount of Mn SOD from pSSS5* cells as compared to those from pDT1.22 cells, which was clearly visible when both the cell types were treated with 100µM paraquat concentration in the cell medium. This comparable increase in Mn SOD in the pSSS5* cells have indeed been confirmed by our recent findings (Chary *et al.*, manuscript in preparation). Also, both pDT1.22 Mn SOD and pSSS5* Mn SOD were cloned from virulent strains of *Enterobacteriaceae*; namely *E.coli*-GC4468 and the *Salmonella typhimurium* virulent 2000 strain, respectively. However, there is a distinct difference in their impact on % survivability at 100µM paraquat concentration. Thus, it is clearly suggestive of the fact that although both the sources for Mn SOD are virulent, there must be a differential regulation of this virulence based on their difference in % survivability. This would in turn again support the fact that Mn SOD from *Salmonella typhimurium* virulent 2000 strain could be more potent.

Thirdly**,** recent studies have shown that *Salmonella typhimurium* has two Cu/Zn periplasmic SODs *versus* only one Cu/Zn SOD in the periplasm of *E. coli* which have an impact on protection of these cells [23], [24], [25], [26] & [27]. This is in addition to the already known constitutive Fe and inducible Mn SODs in their cytoplasm ([21] Carloiz and Touati., 1986). Our present studies also corroborate these findings. As shown in Table 1 and Figs.1A and 1B, when *Salmonella typhimurium* virulent 2000 strain and *E.coli* SOD double-mutant QC779 were grown in LB media containing paraquat concentrations as high as 500µM paraquat, the wild type *Salmonella typhimurium* virulent 2000 strain ***alone*** survived. This finding further supports the fact that the 2 Cu/Zn SODs in *S.typhimurium* could offer an added advantage. Our premise is supported by the recent findings that *S.enterica Servar typhimurium* has a novel protein that is indispensable for virulence and intracellular replication [28].

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Fourthly, the significant role of Mn SOD over Cu/Zn SOD can be extrapolated from several findings in the final analysis. To substantiate this point, a) There are numerous instances especially in Human cells which clearly exhibit the importance of Mn SOD [29], [30], [31], [32], [33] & [34]. b) Cu/Zn SOD null-mutants of *N. crassa* survived in the presence of aerobic conditions, although to a lesser extent than the wild-type *N.crassa* cells. These mutants survived to a far lesser extent in the presence of paraquat [35] & [36]. However, it was not possible to obtain null-mutants of Mn SOD from *N. crassa* (Natvig and Dvorachek, Personal Communication). c) Studies on *E.coli* double mutants of Mn and Fe SOD grown aerobically, in rich medium had no major effect on growth or sensitivity to the superoxide generator, paraquat. However, in minimal medium although aerobic growth was not affected, the sensitivity to paraquat increased, especially in the *sod A* (Mn SOD) mutant. [4] & [21]. This is in spite of the periplasmic Cu/Zn SOD being unaffected in the double-mutants; thus clearly suggesting that Mn SOD is indeed very primordial to an aerobic cell. d) Ultimately, the location of the Mn SOD in the inter-membrane spaces of mitochondria in the eukaryotes and also in the cytoplasm of the prokaryotes allows it to be in close proximity to where respiration occurs. Thus, the release of toxic-free-radicals are immediately detoxified by the readily available presence of Mn SOD which serves as the guardian of the Powerhouse, the mitochondria [37].

Table 1: Effect of Paraquat on Various Cell Strains

PQ Concentration	% Survival			
	S.t. 2000	QC 779	pDT 1.22	pSSS5*
0	100.0	100.0	100.0	100.0
25 µM	96.2	**100.0**	98.9	95.0
50 µM	76.9	4.8	83.3	75.8
100 µM	63.8	**4.8**	**6.9**	**13.9**
250 µM	50.8	0.0	0.0	0.0
500 µM	44.4	0.0	0.0	0.0
1000 µM	0.0	0.0	0.0	0.0

Note: The Table 1 indicates the % survivability of cells in various Paraquat Concentrations for WT, *S. typhimurium* virulent 2000, QC779, pDT1.22 and pSSS5*

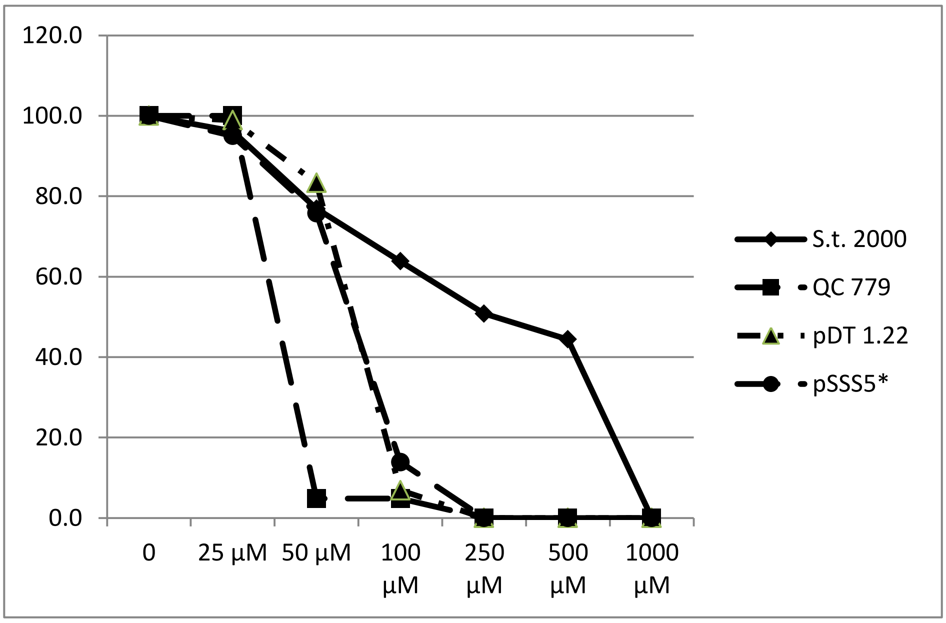

Fig.1A: Represents a Line graph of the data shown in Table 1, in a fashion wherein the values of % survivability of 4 cell types are compared with each other at various Paraquat Concentrations.

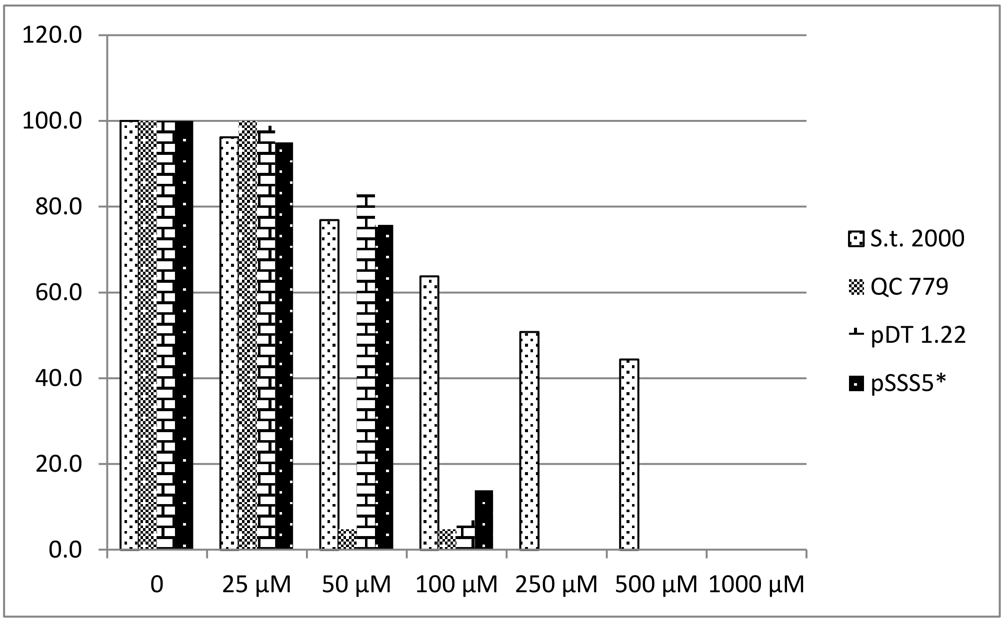

Fig.1B: Represents a Histogram of the data shown in Table 1, in a fashion wherein the values of % survivability of 4 cell types are compared with each other at various Paraquat Concentrations.

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